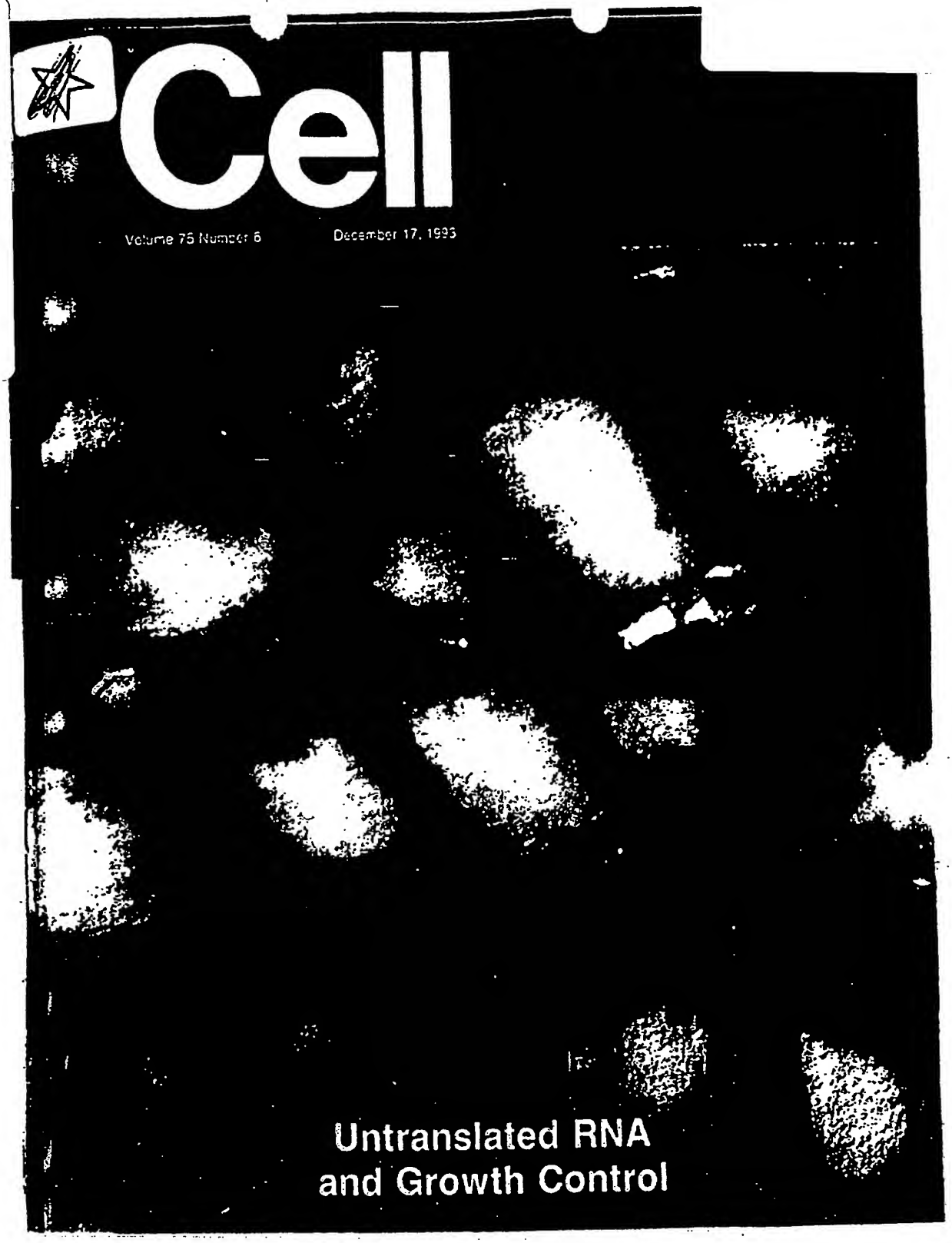
Exhibit B



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Molecular Cloning and Expression of the Fas Ligand, a Novel Member of the Tumor Necrosis Factor Family

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Summary

The Fas antigen (Fas) belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor family, and it mediates apoptosis. Using a soluble form of mouse Fas, prepared by fusion with human immunoglobulin Fc, Fas ligand was detected on the cell surface of a cytotoxic T cell hybridoma, PC60-d10S. A cell population that highly expresses Fas ligand was sorted using a fluorescence-activated cell sorter, and its cDNA was isolated from the sorted cells by expression cloning. The amino acid sequence indicated that Fas ligand is a type II transmembrane protein that belongs to the TNF family. The recombinant Fas ligand expressed in COS cells induced apoptosis in Fas-expressing target cells. Northern hybridization revealed that Fas ligand is expressed in activated splenocytes and thymocytes, consistent with its involvement in T cell-mediated cytotoxicity and in several nonlymphoid tissues, such as testis.

Introduction

Mammalian development is tightly regulated not only by the proliferation and differentiation of cells but also by cell death (Ellis et al., 1991; Raff, 1992). The cell death that occurs during development or tissue turnover is called programmed cell death, most of which proceeds via apoptosis (Walker et al., 1988; Wyllie et al., 1980). Apoptosis is morphologically distinguished from necrosis, which occurs during the accidental cell death caused by physical or chemical agents: During apoptosis, the cytoplasm of the affected cells condenses, and the nucleus also condenses and becomes fragmented. At the final stage of apoptosis, the cells themselves are fragmented (apoptotic bodies) and are phagocytosed by neighboring macrophages and granulocytes (Raff, 1992; Walker et al., 1988; Wyllie et al., 1980). Apoptosis occurs not only during programmed cell death, but also during the death process induced by some cytotoxic T cells, such as tumor necrosis factor (TNF) or lymphotoxin (LT) (Cohen et al., 1992; Golstein et al., 1991).

The Fas antigen (Fas) is a cell surface protein of relative molecular weight (Mr) 45 kd and carries a single transmembrane domain (Itoh et al., 1991; Oehm et al., 1992; Watanabe-Fukunaga et al., 1992b). Fas is a member of the TNF/nerve growth factor receptor family, which includes two TNF receptors (type I, β or 55 kd; type II, α or 75 kd), the low-affinity nerve growth factor receptor, and CD40, CD27, CD30, and OX40 (Nagata, 1993). Members of this family are homologous as to sequences in the extracellular regions. A high level of Fas mRNA expression has been detected in various tissues, such as the thymus, liver, lung, heart, and ovary of adult mice (Watanabe-Fukunaga et al., 1992b). Some monoclonal antibodies (anti-Fas or anti-APO-1 antibodies) against human or mouse Fas work as agonists and induce apoptosis of the cells expressing Fas, in vitro and in vivo (Itoh et al., 1991; Ogasawara et al., 1993; Trauth et al., 1989). These results suggested that Fas is a receptor for an unidentified ligand and transduces the apoptotic signal into cells.

The genetic and molecular analyses of the mouse Fas chromosomal gene indicated that Fas is encoded by the gene at the locus of the mouse lymphoproliferation mutation *lpr* (Adachi et al., 1993; Watanabe-Fukunaga et al., 1992a), which is a natural, autosomal, and recessive mutation (Cohen and Eisenberg, 1991). Since mice carrying mutations homozygous at the *lpr* locus have lymphadenopathy and autoimmune disease (Cohen and Eisenberg, 1991), it is likely that Fas is involved in the development of T cells. Accordingly, Fas is expressed in most thymocytes of the wild type, but not in *lpr* mice (Ogasawara et al., 1993). Immature T cells are killed by apoptosis at least in two steps during development in the thymus (Ramsdell and Fowlkers, 1990). Those T cells carrying T cell receptors that do not recognize self MHC antigens as a restriction element are killed or neglected, while the T cells recognizing the self antigens are killed by a process called negative selection. Analysis of thymic T cell development in wild-type and *lpr* mice has suggested that the neglected thymocytes escape from apoptosis in the thymus, then go to the periphery in *lpr* mice (Zhou et al., 1993). In addition to being expressed in thymocytes, Fas is expressed in activated mature T cells (Trauth et al., 1989). Russell et al. (1993) have suggested a role of Fas-mediated apoptosis in the induction of peripheral tolerance, in the antigen-stimulated suicide of mature T cells, or both.

Fas seems to be involved not only in the development and turnover of T cells, but also in cytotoxic T cell (CTL)-mediated apoptosis. Rouvier et al. (1993) showed that the presence of Fas on target cells was required for their lysis by a CTL hybridoma (PC60-d10S [d10S]) and more generally for calcium-independent cytolysis by CTLs. These results suggested that CTL cells express the Fas ligand on their surface and that the interaction of Fas ligand with Fas on target cells induces apoptosis in target cells.

To understand the role and mechanism of Fas-mediated apoptosis in various systems, it is essential to identify the Fas ligand. In this study, we isolated a cDNA for the Fas

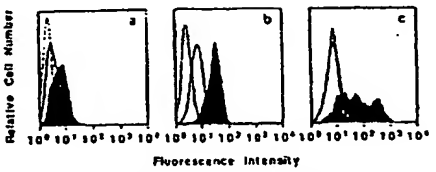

Figure 1. Flow Cytometry of d10S and COS Cells Transfected with Fas Ligand cDNA

(a and b) Flow cytometry of d10S and d10S-2 cells. The d10S cells were sorted twice by FACS to obtain d10S-2 cells, as described under Experimental Procedures. The d10S cells (1×10^6) (a) or d10S-2 cells (1×10^6) (b) were stained with biotinylated mFas–Fc and PE-conjugated streptavidin before (stippled area) or after (closed area) exposure to PMA and ionomycin for 4 hr. Each sample was washed and analyzed by flow cytometry. The staining profile with PE-conjugated streptavidin alone is also shown (open area).

(c) Flow cytometry of COS cells transfected with pTN24-15. Monkey COS cells (4×10^6) were transfected with pTN24-15. The cells were detached from the plates with EDTA 72 hr later, stained with biotinylated mFas–Fc and PE-labeled streptavidin (closed area), or PE-labeled streptavidin alone (open area), and analyzed by flow cytometry. Control COS cells were transfected with the empty vector, and their staining profile with biotinylated mFas–Fc and PE-conjugated streptavidin is also shown (stippled area).

ligand expressed in d10S cells. The structural analysis of the cDNA indicated that the Fas ligand is a type II membrane protein homologous to members of the TNF family, which includes TNFα, TNFβ, LTβ, and the ligands for CD40, CD27, and CD30.

Results

Molecular Cloning of the Fas Ligand

To identify the Fas ligand, we prepared a chimeric protein (mFas–Fc) consisting of the extracellular region of the mouse Fas antigen fused to the hinge, CH2, and CH3 domains of human immunoglobulin γ1 heavy chain (Arulto et al., 1990). As a control, a chimeric molecule (hTNFRβ–Fc) between the extracellular region of human TNF receptor β (p55) and the Fc region of human immunoglobulin G1 was prepared by a similar method.

The T cell hybridoma, d10S, is cytotoxic against cells expressing Fas, but not against those which do not (Rouvier et al., 1993). The cytotoxic activity of d10S was inhibited by mFas–Fc, but not by hTNFRβ–Fc (see below; T. S. and S. N., submitted). When d10S cells were stained with biotinylated mFas–Fc followed by phycoerythrin (PE)-conjugated streptavidin, there was a small shift in a flow cytometric profile (Figure 1a). Stimulation of d10S cells with phorbol myristic acetate (PMA) and ionomycin enhances their cytotoxic activity (Rouvier et al., 1993). Accordingly, this treatment greatly increased the expression of a protein which binds mFas–Fc in d10S cells (Figure 1a). On the other hand, d10S cells were not significantly stained with biotinylated hTNFRβ–Fc, indicating that mFas–Fc specifically bound to the Fas ligand (data not shown).

To facilitate cDNA cloning, a population of d10S cells, which expressed large amounts of the Fas ligand, was selected by FACS using biotinylated mFas–Fc and PE-conjugated streptavidin, as described under Experimental Procedures. To exclude the possible enrichment of Fcγ receptor–positive population, cells were also stained with fluorescein isothiocyanate (FITC)–labeled hTNFRβ–Fc, and a cell population (about 0.3%–0.5%) that stained intensely with mFas–Fc but not with hTNFRβ–Fc was sorted. Two rounds of sorting of d10S cells (d10S-2) significantly enriched the cell population that highly expressed the Fas ligand, under both unstimulated and stimulated conditions (Figure 1b).

A cDNA library (~2.2×10^6 clones) in a mammalian expression vector (pCEV4) (Itoh et al., 1991) was constructed with mRNA from the PMA and ionomycin-stimulated d10S-2 cells. Plasmid DNA from the whole library was introduced into COS cells. The transfected COS cells were allowed to bind mFas–Fc and were then treated with a chemical cross-linker (Harada et al., 1990). The cells cross-linked with mFas–Fc were enriched by panning, as described under Experimental Procedures. This procedure (enrichment by panning and amplification in Escherichia coli [E. coli]) was repeated four times, and plasmid DNAs were prepared from individual bacterial clones after the fourth round. Clones (12 out of 48) contained inserts of more than 1.0 kb, and they were individually introduced into COS cells. When these cells were stained with biotinylated mFas–Fc and analyzed by flow cytometry, five of them were positive. A typical result with COS cells transfected with pTN24-15 is shown in Figure 1c. In contrast, COS cells transfected with the empty vector were not stained with biotinylated mFas–Fc. Furthermore, COS cells transfected with pTN24-15 did not significantly bind biotinylated hTNFRβ–Fc (data not shown).

Structure of the Fas Ligand

Restriction enzyme mapping of the inserts of the five positive clones indicated that they overlapped. One clone containing a 1.6 kb insert, pTN24–15, was therefore randomly selected and further characterized. The nucleotide sequence of pTN24-15 and its predicted amino acid sequence are presented in Figure 2. The cDNA consists of 1623 nt, and there is only one long open reading frame. The initiation site was tentatively assigned to the ATG codon at nucleotide positions 74–76, although the nucleotide sequence surrounding this initiation site does not conform well with the consensus sequence (CCA/GCCATGG) proposed by Kozak (1991). The open reading frame ends at the termination codon TAA at positions 908–910 and thus codes for a protein of 278 amino acids with a calculated Mr of 31,138 and an isoelectric point of 9.53. The first 77 amino acids of this protein are extremely rich in proline residues. Although it lacks a signal sequence at the N-terminus, its hydropathy analysis indicated that the proline-rich region is followed by 22 hydrophobic amino acids that presumably function as a transmembrane anchor. The lack of a signal sequence and the presence of an internal hydrophobic domain suggested that the Fas ligand is a type II transmembrane protein. The putative extracellular domain in its carboxyl region consists of 179 amino acids and contains four potential N-glycosylation sites (N–X–S/T).

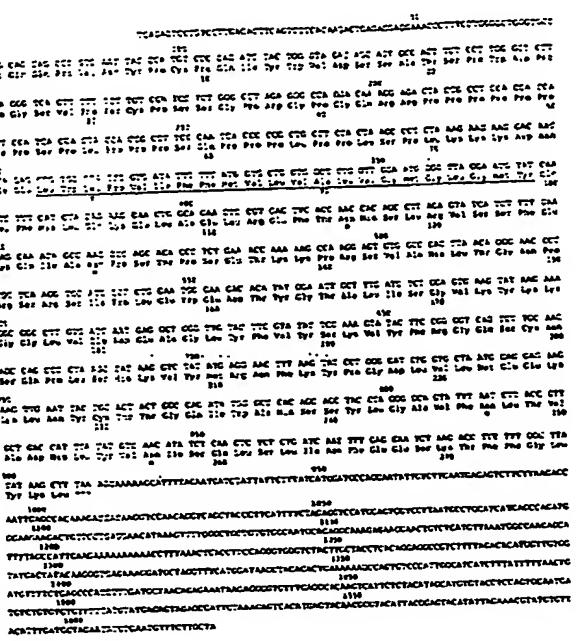

Figure 2. Nucleotide Sequence and Predicted Amino Acid Sequence of Rat Fas Ligand cDNA

The numbers above and below each line refer to the nucleotide position and amino acid positions, respectively. The putative transmembrane domain is underlined and four potential N-linked glycosylation sites (N–X–S/T) are indicated by asterisks.

The d10S cell line used to prepare the cDNA library is a hybrid between rat and mouse cell lines (Conzelmann et al., 1982). To determine the origin of the cloned Fas ligand cDNA, two sets of oligonucleotides containing sequences for the 3′ noncoding region of pTN24-15 (nucleotides from positions 1006–1025 and 1305–1324 for the forward primer and 1327–1346 and 1543–1562 for the reverse primer) were synthesized. When genomic DNAs from the rat and mouse spleen were analyzed by polymerase chain reaction, only rat chromosomal DNA gave bands of the predicted sizes of 341 bp and 258 bp (data not shown). Furthermore, Northern hybridization with pTN24-15 as probe under high stringency gave a band of 2 kb in rat but not mouse RNA (see below, T. T., T. S., and S. N., unpublished data). These results indicated that the cloned cDNA sequence was derived from the rat genome present in the d10S hybrid cell line.

Biochemical and Biological Properties of the Fas Ligand

The Fas ligands expressed in d10S cells and in the COS cells transfected with pTN24-15 were biochemically characterized by immunoprecipitation using mFas–Fc. The mFas–Fc specifically precipitated protein(s) of Mr 38–42 kd from biotinylated cell surface proteins of the d10S-12 cell line (sorted 12 times by FACS) (Figure 3; T. S. and S. N., submitted). Similarly, immunoprecipitation of the lysates from COS cells transfected with pTN24-15, but not with the empty vector, gave several bands of Mr 36–43 kd, which differed slightly from the bands detected in d10S-12 cells. The Mr of the Fas ligand expressed in d10S-12 cells or COS cells is larger than that calculated from its amino acid sequence (Mr, 31,138). The difference is probably due to glycosylation of some of the four N-glycosylation sites. This may also explain the slightly different Mr between the natural Fas ligand in d10S cells and the recombinant Fas ligand expressed in COS cells.

To confirm that pTN24-15 codes for the Fas ligand, the cytotoxic activity of the COS cells expressing the recombinant Fas ligand was examined using WR19L transformants (W4) that express mouse Fas (Ogasawara et al., 1993) as target cells. As shown in Figure 4b, COS cells transfected with pTN24-15 induced cytolysis of W4 in a dose-dependent manner, while control COS cells transfected with the empty vector exhibited no cytotoxic activity. The transfected COS cells were at least 10 times more efficient as effectors against W4 cells, based upon the effector/target cells ratio, than were the original d10S cells (Figures 4a and 4b). The Fas ligand expressed in d10S or COS cells could not kill parental WR19L cells (Figure 4). Since WR19L cells are susceptible to killing by TNF (Itoh et al., 1993), this suggests that the Fas ligand cannot induce the death signal through the TNF receptor. To confirm the specificity of the Fas ligand, mFas–Fc or hTNFRβ–Fc was added to the assay mixture. As shown in Figure 4d, the cytotoxic activities of COS cells expressing the recombinant Fas ligand, as well as that of the d10S cells, were almost completely inhibited by 10 μg/ml of mFas–Fc, but not by hTNFRβ–Fc. The supernatant of COS cells transfected with the Fas ligand cDNA also had significant

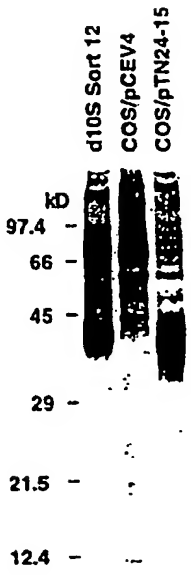

Figure 3. Immunoprecipitation of the Fas Ligand with the mFas–Fc

The d10S cells were sorted 12 times by FACS to obtain the d10S-12 cell line. COS cells were transfected with pCEV4 vector DNA or pTN24-15 carrying Fas ligand cDNA. After 72 hr, transfected COS cells were detached with EDTA. The cell surface proteins of the d10S-12 cell line and the transfected COS cells were biotinylated, lysed in buffer containing NP-40, precleared with hTNFRβ–Fc, and immunoprecipitated with mFas–Fc, as described under Experimental Procedures. The immunoprecipitates were heated at 95°C for 2 min in Laemmli's sample buffer containing 5% 2-mercaptoethanol and resolved by electrophoresis through a 10%–20% gradient polyacrylamide gel in the presence of 0.1% SDS. The proteins were blotted onto a PVDF membrane (Millipore) and detected using the ECL system (Amersham) after staining with streptavidin-conjugated horseradish peroxidase. As size markers, biotin-labeled molecular weight standards (Pharmacia) were electrophoresed in parallel; sizes of standard proteins are shown in kilodaltons.

cytotoxic activity upon W4, but not upon WR19L parental cells (Figure 4c). These results suggest that the recombinant Fas ligand expressed in COS cells can be released into the extracellular fluid, perhaps by cleavage from the cell surface.

We and others have reported that the Fas activated by agonistic anti-Fas antibodies mediates apoptosis (Itoh et al., 1991; Ogasawara et al., 1993; Trauth et al., 1989). The recombinant Fas ligand expressed on COS cells also induced apoptosis. In Figure 5, the chromosomal DNA was prepared from W4 cells after various periods of incubation with COS cells that were transfected either with pTN24-15 or with the empty vector pCEV4. COS cells transfected with pTN24-15 induced fragmentation of the chromosomal DNA of W4 cells in a step ladder fashion that is characteristic of apoptosis (Compton, 1992). The DNA ladder was

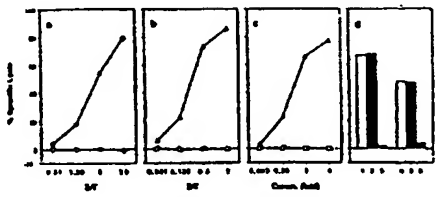

Figure 4. Cytotoxic Activity of d10S Cells and the Recombinant Fas Ligand Expressed in COS Cells

(a) Cytotoxic activity of d10S cells. The cytotoxic activity was assayed in duplicate using ^{51}Cr-labeled W4 (closed circle) or WR19L cells (open circle), as described under Experimental Procedures.

(b) Cytotoxic activity of the recombinant Fas ligand expressed in COS cells. COS cells were transfected with the pCEV4 vector (closed circle) or pTN24-15 DNA (closed triangle, open circle). At 72 hr after transfection, cytotoxicity of the transfected COS cells using W4 (closed circle, closed triangle) or WR19L cells (open circle) as target cells is shown.

(c) Cytotoxic activity of the soluble recombinant Fas ligand produced by COS cells. COS cells were transfected with pCEV4 (closed circle) or pTN24-15 (closed triangle, open circle). At 18 hr after transfection, the medium was changed to serum-free DMEM, and cells were incubated for 48 hr. The culture supernatant of the transfected cells was filtrated through 0.22 μm filter, concentrated 8-fold, and then added to the target cells. W4 cells (closed circle, closed triangle) or WR19L cells (open circle) were used as target cells. One-fold concentration corresponds to the neat supernatant of COS cells.

(d) Inhibition of the cytotoxic activity of the Fas ligand with mFas–Fc. The cytotoxic activity of the activated d10S cells at an effector/target (E/T) ratio of 10 (lanes 1–3) or COS cells transfected with pTN24-15 at an E/T ratio of 0.2 (lanes 4–6) was determined using ^{51}Cr-labeled W4 cells as target cells. No chimeric proteins (open bar), 10 μg/ml of hTNFRβ–Fc (hatched bar), or mFas–Fc (closed bar) was added to the reaction mixture.

observed within 1 hr of incubation, and most of it was degraded into nucleosome-sized fragments after 2 hr. Such fragmentation was not observed in W4 cells incubated with COS cells transfected with pCEV4 (Figure 5). Moreover, the activity of the recombinant Fas ligand was seen only in W4 cells, but not in WR19L cells (data not shown).

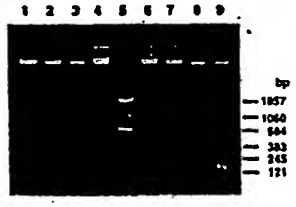

Figure 5. DNA Fragmentation Induced by the Recombinant Fas Ligand

COS cells were transfected with pCEV4 vector (lanes 1–4) or the Fas ligand expression plasmid pTN24-15 (lanes 6–9). At 48 hr after transfection, W4 cells were added to the transfected COS cells and incubated at 37°C. Before incubation (lanes 1 and 6) and after incubation for 1 hr (lanes 2 and 7), 2 hr (lanes 3 and 8), or 3 hr (lanes 4 and 9), total DNA was prepared from the nonadherent cells and resolved on a 1.0% agarose gel in the presence of 0.5 μg/ml of ethidium bromide. For size markers (lane 5), BamHI-digested and MvaI-digested pBR322 was electrophoresed in parallel; sizes of the DNA fragments are given in base pairs.

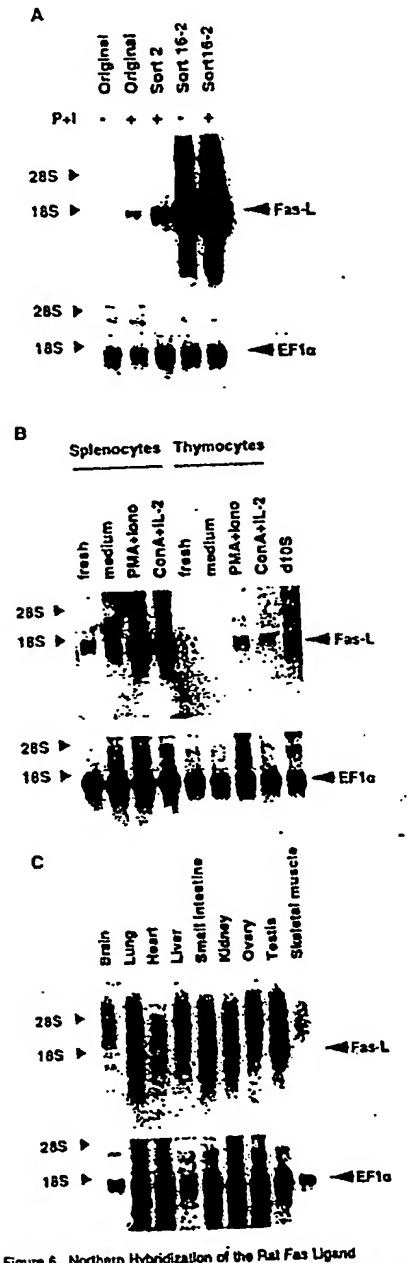

Figure 6. Northern Hybridization of the Rat Fas Ligand

(A) Expression of the Fas ligand mRNA in the d10S cell line and its sublines. Poly(A) RNA was prepared from the d10S (original), d10S-2 (sorted twice), or d10S-16 (sorted 16 times) cells before (minus sign) or after (plus sign) stimulation with PMA and ionomycin. One microgram of mRNA per lane was electrophoresed on a 1.5% agarose gel and analyzed by Northern hybridization using the ^{32}P-labeled Fas ligand cDNA (Fas-L, upper panel) or human EF-1α cDNA (lower panel). The positions of 18S and 28S rRNAs are indicated on the left.

(B) Expression of the Fas Ligand in Rat Splenocytes and Thymocytes. Freshly isolated rat splenocytes or thymocytes were cultured at 37°C for 8 hr in the medium alone or in that containing 10 ng/ml PMA and 500 ng/ml ionomycin or 5 μg/ml concanavalin A and 10 ng/ml human interleukin 2. Poly(A) RNA, 2.0 μg per lane, was subjected to Northern hybridization with the rat Fas ligand cDNA (Fas-L, upper panel) or human EF-1α cDNA as a probe (lower panel).

(C) Expression of the Fas ligand mRNA in Rat Tissues. Poly(A) RNA was prepared from the indicated rat tissues, and 2.0 μg RNA per lane was analyzed by Northern hybridization using rat Fas ligand cDNA (Fas-L, upper panel) and human EF-1α cDNA (lower panel) as a probe.

Expression of Fas Ligand mRNA

The original and sorted d10S cell lines, rat splenocytes and thymocytes, and various rat tissues were examined by Northern blot hybridization using the Fas ligand cDNA as a probe. As shown in Figure 6A, poly(A) RNA from the original d10S cells showed a weakly hybridizing band of 2 kb. The intensity of this band increased considerably after the d10S cells were activated with PMA and ionomycin. The d10S-2 cell line, which was sorted twice by FACS, expressed about four times more Fas ligand mRNA than did the original d10S after stimulation with PMA and ionomycin, which is consistent with the results of the flow cytometry (see Figure 1). Recently, we obtained a d10S-16 cell line (sorted 16 times) that exhibited about 100 times higher staining intensity with mFas-Fc and 100 times higher cytotoxic activity than did the original d10S cells (T. S. and S. N., submitted). Accordingly, d10S-16 cells had about 100 times more Fas ligand mRNA than did the original d10S cells (Figure 6A). Thus, the increased expression of Fas ligand mRNA correlated well with the increased staining intensity with mFas-Fc and the cytotoxic activity.

As shown in Figure 6B, fresh rat splenocytes weakly expressed Fas ligand mRNA. When splenocytes were stimulated with PMA and ionomycin, or concanavalin A and interleukin 2, for 8 hr, Fas ligand mRNA expression was greatly induced. No expression or little expression of the Fas ligand mRNA was detected in rat thymocytes. However, it was also induced by PMA and ionomycin, or concanavalin A and interleukin 2, although the expression levels were much less pronounced as compared with those seen in splenocytes. Among various rat tissues, the testis gave an intensely hybridizing band of about 2 kb for the Fas ligand mRNA (Figure 6C). Moderate or weak signals were also observed in the small intestines, kidney, and lung, whereas no other tissues significantly expressed Fas ligand mRNA. All RNA samples were intact since rehybridization of the same membrane with human elongation factor 1α (EF-1α) cDNA probe (Uetsuki et al., 1989) revealed a 1.8 kb band in all lanes.

Discussion

Many cytokine receptor systems mediate the proliferation of cells, the differentiation of cells, or both. Some types

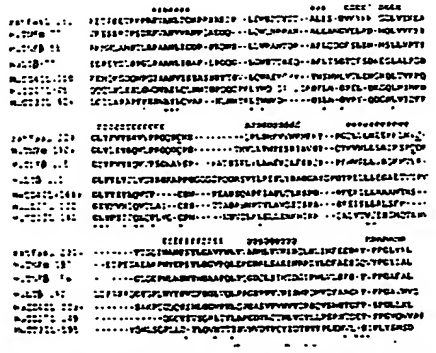

Figure 7. Alignment of the Amino Acid Sequence of the TNF Family Members

The C-terminal amino acid sequence of the rat Fas ligand was aligned with the corresponding amino acid sequences of the other members of the TNF family, beginning at the N-terminus of mature soluble human TNFα. Numbers in brackets indicate the positions from the N-terminus of the primary translation products. Several gaps have been introduced to optimize the alignment. Amino acids identical in more than five members are indicated by asterisks. The amino acids of favored substitutions in more than four members are shown in bold letters. Favored amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; and F, Y, and W. The extent of the β sheet regions a–h of TNFα (Eck and Sprang, 1989) is shown at the top of each line. The cysteine residues found in the corresponding positions of the Fas ligand and TNFα are underlined.

of apoptosis also seem to be regulated by cytokines and their receptors (Nagata, 1993). We previously suggested that Fas is a receptor for an unknown death factor and mediates apoptosis. In this report, the cDNA for the death factor, or the Fas ligand, was isolated from a CTL cell line.

The Fas belongs to the TNF/nerve growth factor receptor family, which includes two TNF receptors, the low affinity nerve growth factor receptor, and CD40, CD30, and CD27 (Itoh et al., 1991; Nagata, 1993). TNFα exists either as a type II transmembrane protein or a soluble cytokine (Kriegler et al., 1988). The ligands for CD40, CD27, and CD30 have been identified as type II transmembrane proteins related to TNF (Armitage et al., 1992; Smith et al., 1993; Goodwin et al., 1993). Recently, it was demonstrated that LTβ, which can associate with TNFβ (LTα) on the cell surface, is also a member of the TNF family (Browning et al., 1993). The amino acid sequence of the Fas ligand is clearly homologous to those members of the TNF family (Figure 7). Homologous regions are restricted to the carboxyl region; that is, the extracellular domain that interacts with the receptor. A pairwise comparison of the amino acid sequences in this region indicated that the Fas ligand is more similar to TNFα, TNFβ, and LTβ than to ligands for CD40, CD27, and CD30. The high homologies of the Fas ligand with TNFα and TNFβ (27.6% and 28.7% identity, respectively) are comparable to that between TNFα and TNFβ (29.6% identity) (Pennica et al., 1984). Despite this similarity, the cloned rat Fas ligand could neither bind to the 55 kd human TNF receptor nor activate the mouse TNF receptor in WR19L cells.

TNFα and TNFβ function as trimers (Smith and Baglioni, 1987). X-ray diffraction analyses of TNFα and TNFβ have indicated that each monomer of these cytokines forms an elongated, antiparallel β-pleated sheet sandwich with a jelly roll topology (Eck and Sprang, 1989; Eck et al., 1992; Jones et al., 1989; Banner et al., 1993). As shown in Figure 7, the conserved amino acids among the Fas ligand, TNFα, and TNFβ were found mainly within the β strands, suggesting the Fas ligand has a similar structure to TNFα and TNFβ. Furthermore, in TNFα, a disulfide bridge links the loop connecting β strands c and d with that connecting β strands e and f (Eck and Sprang, 1989). The two cysteine residues in the extracellular domain of the Fas ligand were found at the corresponding positions (Figure 7), suggesting that these cysteine residues are connected by a disulfide bond similar to that found in TNFα. These structural similarities of the Fas ligand with TNFα suggest that the Fas ligand also exists as a trimer. The original anti-Fas or anti-APO-1 antibody works as an agonist that induces apoptosis by binding to Fas (Itoh et al., 1991; Trauth et al., 1989). The anti-Fas antibody is an immunoglobulin M (Yonehara et al. 1989), which is an immunoglobulin pentamer, whereas the anti-APO-1 antibody is an immunoglobulin G_3 (Trauth et al., 1989), which tends to aggregate. The $F(ab')_2$ fragment of the anti-APO-1 antibody and its isotypes have little cytotoxic activity (Dhein et al., 1992). However, these divalent anti-APO-1 antibodies induced apoptosis when they were cross-linked by second antibodies. These results indicate that dimerization of Fas is insufficient to transduce the apoptotic signal, and they are consistent with a trimeric structure for the Fas ligand.

Although Rouvier et al. (1993) could not detect Fas ligand activity in the culture medium of the d10S cell line, we detected Fas-dependent cytotoxic activity in the supernatant of COS cells transfected with a Fas ligand expression plasmid. Moreover, the medium conditioned with the d10S-16 cells, which expresses about 100 times more Fas ligand mRNA than the original d10S cell line (Figure 6A), was significantly cytotoxic against cells expressing Fas (T. S. and S. N., unpublished data). These results suggest that overexpression of the Fas ligand on the cell surface causes shedding of the protein, as found in TNF (Kriegler et al., 1988; Perez et al., 1990), and the soluble form of the Fas ligand actively triggers apoptosis by binding to Fas. A large amount of soluble TNF is present in the serum of individuals suffering from septic shock caused by bacterial endotoxins or in the serum of cancer patients (Beutler and Cerami, 1986; Old, 1987). Similarly, production of the soluble Fas ligand may accompany some human diseases. Since administration of agonistic anti-Fas antibody into mice causes a phenotype similar to that of fulminant hepatitis (Ogasawara et al., 1993), it will be of interest to examine the involvement of the soluble Fas ligand in human fulminant hepatitis.

Various effector molecules are thought to be involved in CTL-mediated cytotoxicity (Apasov et al., 1993; Golstein et al., 1991; Podack et al., 1991). One well characterized pathway is the perforin-mediated cytolysis of target cells,

which is dependent on Ca^{2+}. It is also suggested that the soluble or membrane-bound form of TNF or LT is involved in various target cell systems. Recently, Rouvier et al. (1993) demonstrated that the cytotoxic activity of CTL hybridoma d10S depends on the expression of Fas on target cells. Here, we confirmed by generating cytotoxicity with the recombinant Fas ligand that this cytotoxicity is mediated by interaction between the Fas ligand on d10S cells and Fas on target cells. Furthermore, the cytotoxic activity of transfectants of COS (a fibroblast-like kidney-derived cell line) strongly argues that the cytotoxicity mediated by the Fas–Fas ligand system requires no other molecules preferentially expressed in activated lymphocytes (such as perforin or granzymes). Similar Fas-dependent cytotoxicity, which is alloantigen-specific and Ca^{2+}-independent, has been observed in peritoneal exudate lymphocytes (Rouvier et al., 1993). It is likely that the interaction of the relevant MHC on target cells with the T cell receptor on effector cells induces the Fas ligand on the latter, which then bind to Fas on target cells to induce apoptosis (Rouvier et al., 1993). In addition, we detected Fas ligand mRNA in rat splenocytes after stimulation with PMA and ionomycin, or concanavalin A and interleukin 2. These results suggest that the Fas system is a novel and common mechanism of T cell–mediated cytotoxicity. In this regard, it is noteworthy that some $CD4^+$ Th1 cell lines exhibit a cytolytic activity independent of perforin and TNF (Ju, 1991; Ozdemirli et al., 1992). It would be of interest to examine whether or not the Fas system is also involved in these cytolytic activities.

Mice carrying the *lpr* or *gld* mutation develop lymphadenopathy and autoimmune disease (Cohen and Eisenberg, 1991). It has been suggested that the *lpr* and *gld* are mutations of genes encoding an interacting pair of molecules involved in T cell development (Allen et al., 1990). We have previously demonstrated that *lpr* mice have defects in the Fas gene and thus proposed that the Fas ligand is encoded by the gene at the *gld* locus (Watanabe-Fukunaga et al., 1992a). Recent findings that peritoneal exudate lymphocytes from *gld* mice do not show the Fas-dependent cytotoxicity (P. G., unpublished data) support our notion. To confirm this theory, it would be necessary to correlate the chromosomal gene locus of mouse Fas ligand with the *gld* locus and to examine the abnormality of the chromosomal gene for the Fas ligand in *gld* mice. The *lpr* or *gld* mice are thought to have defects in the thymic development of T cells (Cohen and Eisenberg, 1991). We previously suggested that the expression of the Fas in thymocytes and the Fas ligand in thymic stromal cells control the development of T cells (Watanabe-Fukunaga et al., 1992a). Although most thymocytes express Fas (Ogasawara et al., 1993), we could not detect the Fas ligand transcript in the thymus except for weak expression in activated thymocytes. It is possible that the Fas ligand is expressed only transiently in a limited number of stromal cells in the thymus. The availability of Fas ligand cDNA and mFas–Fc fusion protein would facilitate the identification of such cells by in situ hybridization and immunohistochemistry.

The Fas is expressed not only in the cells of the immune system, but also in the liver, lung, ovary, and heart (Watanabe-Fukunaga et al., 1992b). The physiological functions of Fas in these tissues are not clear, and no developmental abnormalities of these tissues have been noticed in *lpr* nor *gld* mice. There was no major expression of Fas ligand mRNA in the aforementioned tissues, although, for instance, the lung expresses Fas and some detectable level of Fas ligand mRNA. On the other hand, the Fas ligand was expressed in the testis, where no apparent expression of Fas was previously detected (Watanabe-Fukunaga et al., 1992b). The testicular seminiferous epithelium is highly proliferative, and many germ cells are programmed to die by apoptosis by interaction with Sertoli cells (Allan et al., 1992; Miething, 1992). Whether the Sertoli cells express the Fas ligand to kill the germ cells remains to be examined. In any event, these results are consistent with the involvement of the Fas system in various aspects of mammalian development.

Experimental Procedures

Flow Cytometry and Selection of the d10S-2 Cell Line

The expression plasmids for mFas–Fc and hTNFRβ–Fc were constructed with mouse *Fas* gene (Watanabe-Fukunaga et al., 1992b) or human TNFR expression plasmid p55TNFr-HG1 (Loetscher et al., 1991) and human immunoglobulin gene in plasmid pMH4 (Nishimura et al., 1987). These chimeric proteins were transiently produced in COS cells or in stable transformants of BTS-1 cells (Sedivy et al., 1988) and were homogeneously purified. The details of the production procedure will be described elsewhere (T. S. and S. N., submitted). The mFas–Fc and hTNFRβ–Fc were biotinylated using sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS–LC–biotin, Pierce) according to the protocol of the manufacturer. To prepare FITC-conjugated human TNFRβ–Fc, 1 mg of protein was mixed with 20 μg of FITC in 1 ml of 50 mM sodium carbonate buffer (pH 9.5). After incubation at room temperature for 4 hr, free FITC was removed by Sephadex G-25M column chromatography. For flow cytometry, d10S or transfected COS cells were washed with staining solution (phosphate-buffered saline [PBS] containing 2% fetal calf serum [FCS] and 0.02% NaN_3). Cells ($\sim 1 \times 10^6$) were first incubated on ice for 10 min in 50 μl of staining solution containing 5 μg/ml rat anti-mouse FcγII receptor antibody (Pharmingen). Fifty microliters of biotinylated mFas–Fc (10 μg/ml) was added to the reaction mixture and incubated on ice for 30 min. After washing with staining solution, the cells were stained on ice for 30 min with PE-conjugated streptavidin (25-fold dilution, Becton-Dickinson) in 100 μl of staining solution. Cells were washed with staining solution and analyzed by flow cytometry with a FACScan (Becton-Dickinson).

A subline of the d10S cell line that stained intensely with mFas–Fc was selected by repetitive FACS sorting: In brief, 1×10^7 to 3×10^7 d10S cells were stained with FITC-conjugated hTNFRβ–Fc and biotinylated mFas–Fc followed by PE-conjugated streptavidin, as described above, and sorted using a FACStar (Becton-Dickinson). The cells providing the highest levels of PE-fluorescence signal (top 0.3%–0.5%), but not significantly stained by FITC-hTNFRβ–Fc, were collected and expanded in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and 50 mM 2-mercaptoethanol.

Cytotoxicity Assays

Cytotoxicity was assayed essentially as described previously (Rouvier et al., 1993). WR19L or W4 cells (1×10^6) were incubated for 2 hr at 37°C with 20 μCi of [^{51}Cr] sodium chromate (Amersham) in 100 μl of RPMI1640 containing 10% FCS. After washing with medium, these cells were used as the target. Transfection of COS cells with the pTN24-15 or the control vector was performed by DEAE–dextran method, as described previously (Fukunaga et al., 1990). The ^{51}Cr-labeled target cells (1×10^4) were mixed with original d10S cells or transfected COS cells at various ratios in round-bottomed microtiter plates in a total volume of 200 μl. The plates were centrifuged at 700 rpm for 2 min and incubated for 4 hr at 37°C. The plates were then

centrifuged at 1200 rpm for 5 min, and 100 μl aliquots of the supernatants were assayed for radioactivity using a γ-counter. The spontaneous release of ^{51}Cr was determined by incubating the target cells with the medium alone, whereas the maximum release was determined by adding Triton X-100 to a final concentration of 0.1%. The percentage of specific ^{51}Cr release was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{experimental } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}}{\text{maximum } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}}$$

The spontaneous release of ^{51}Cr was routinely 8%–10% of the maximum release.

Construction of the cDNA Library

The d10S-2 cells were grown up to 2 × 10^6 cells/ml in DMEM containing 10% FCS and were then stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin at 37°C for 3 hr. Total cellular RNA was isolated using guanidine isothiocyanate–phenol acid (Chomczynski and Sacchi, 1987), and poly(A) RNA was selected by two cycles of oligo(dT)–cellulose column chromatography. Double-stranded cDNA primed with a random hexamer or oligo(dT) was synthesized as described (Itoh et al., 1991). A BstXI adaptor was added to the cDNA, which was size-fractionated by electrophoresis on a 1% agarose gel. Those cDNAs larger than 1.5 kb were recovered and ligated to BstXI-digested pCEV4. E. coli DH10B cells (GIBCO BRL) were transformed with the ligated DNA by electroporation (Dower et al., 1988). About 1.0 × 10^6 independent clones from an oligo(dT)-primed cDNA library were mixed with 1.3 × 10^6 clones from a random hexamer-primed cDNA library and were used to transfect COS7 cells.

Enrichment of cDNA Clones for the Fas Ligand by Panning

COS7 cells were transfected with plasmid DNA by electroporation (Potter et al., 1984). In brief, 5 × 10^6 COS cells were washed with K-PBS$^-$ (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.46 mM KH_2PO_4), and suspended in 0.4 ml of K-PBS$^-$ supplemented with 5 mM $MgCl_2$ (K-PBS$^+$). Plasmid DNA (40 μg) dissolved in 0.4 ml of K-PBS$^+$ was added to the cell suspension and incubated on ice for 10 min. Cells were then exposed to a 230 v pulse with a capacitance of 960 μF and returned to the ice. After 10–15 min, the cell suspensions were diluted with 5 ml of cold serum-free DMEM and incubated for 30 min at room temperature. The cells were then plated on 2 10-cm dishes and cultured for 60 hr at 37°C in DMEM containing 10% FCS. A total of 1.2 × 10^8 COS cells were transfected as above and cultured in 96 10-cm plates. The cells were detached from the plates by incubating at 37°C for 30 min in 5 ml per plate of PBS–EDTA–NaN_3 (PBS containing 0.5 mM EDTA and 0.02% NaN_3). The detached cells were resuspended to a concentration of 5 × 10^6 to 7 × 10^6 cells per milliliter in PBS–EDTA–NaN_3 containing 3 mg/ml of bovine serum albumin and 2.5 μg/ml anti-mouse FcγII receptor antibody. After incubation on ice for 10 min, mFas–Fc was added to a final concentration of 4 μg/ml and incubated on ice for 60 min. The cells were washed with ice-cold PBS and suspended to 5 × 10^6 to 7 × 10^6 cells per milliliter in PBS containing 50 mM HEPES buffer (pH 8.3) and 0.2 mM bis(sulfosuccinimidyl) suberate (BS^3, Pierce). After incubation on ice for 30 min, 1 M Tris–HCl (pH 8.0) was added to a final concentration of 50 mM and incubated on ice for 10 min. After washing with PBS, cells were suspended in 30 ml of PBS–EDTA–NaN_3 containing 3 mg/ml bovine serum albumin and passed through Nylon mesh (pore size, 100 μm) to remove aggregates. The cells were then distributed into 30 10-cm panning plates that were coated with goat anti-human immunoglobulin G Fc (Cappel). After incubation for 2 hr at room temperature, nonadherent cells were removed by gentle washing with PBS–EDTA–NaN_3 containing 3 mg/ml bovine serum albumin, and the extrachromosomal DNA was extracted from the adherent cells as described (Itoh et al., 1991). Electroporation of E. coli with the DNA obtained from the first round of panning yielded 4.1 × 10^6 colonies, from which plasmid DNA was prepared, and was used to transfect 9.8 × 10^7 COS cells (50 10-cm plates). The second panning using 30 plates and DNA preparation from the adherent cells proceeded as described above. Transformation of E. coli with the recovered plasmid DNA yielded 8 × 10^6 clones, and the third panning using 30 plates was performed by transfecting 4 × 10^7 COS cells (in 10 plates). With the plasmid DNA recovered from the adherent cells, E. coli was transformed to produce 3.6 × 10^6 clones, which were subjected to a fourth panning, using 1 × 10^7 COS cells (in 25 plates) and 10 panning plates.

DNA Sequence Analysis and Northern Hybridization

The DNA sequence was determined using a DNA sequencer (model 370A; Applied Biosystems) and a Taq DyeDeoxy cycle sequencing kit from Applied Biosystems. For Northern hybridization, poly(A) RNA was prepared from d10S and its derivatives and various rat tissues and cells using a mRNA isolation kit from Pharmacia. RNA was denatured at 65°C for 5 min in 50% formamide, electrophoresed through a 1.5% agarose gel containing 6.5% formaldehyde, and transferred to nitrocellulose or nylon membranes (Schleicher & Schuell). The probe was a 0.9 kb DNA fragment containing the sequence from 43 to 967 of pTN24-15, prepared by PCR, and labeled with ^{32}P using a random primer labeling kit (Boehringer, Mannheim). A ^{32}P-labeled 1.8 kb BamHI fragment of human EF-1α cDNA (Uetsuki et al., 1989) was used as a control probe DNA. Hybridization proceeded as described (Sambrook et al., 1989) under high stringency.

Fragmentation of Chromosomal DNA

COS cells (8 × 10^5) in 24 well plates were transfected with 1.0 μg of pTN24-15 using DEAE–dextran as described (Fukunaga et al., 1990). WR19L cells or W4 cells (Ogasawara et al., 1993) (2 × 10^6) were added to the wells 48 hr after transfection and incubated in RPMI1640 medium containing 10% FCS at 37°C. Nonadherent cells were collected, and chromosomal DNA was prepared as described (Laird et al., 1991).

Surface Labeling and Immunoprecipitation

The cell surface proteins of d10S-12 cells or COS cells transfected with pTN24-15 were biotinylated using D-biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester (biotin–CNHS–ester, Boehringer-Mannheim) as described (Meier et al., 1992). The cells (7.5 × 10^6 cells) were lysed by incubating on ice for 30 min in 1 ml of lysis buffer (1% NP-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM [p-aminophenyl]methanesulfonyl fluoride hydrochloride [APMSF], 1 μg/ml pepstatin, and 1 μg/ml of leupeptin). After centrifugation at 14,000 rpm for 15 min, the supernatant was precleared by incubation on ice for 60 min with 10 μg/ml of hTNFRβ–Fc and then at 4°C for 60 min with a 5% volume of protein A–Sepharose. After removing the protein A–Sepharose, 10 μg of mFas–Fc was added to the supernatant and incubated on ice for 60 min. Protein A–Sepharose (0.01 vol) was added to the mixture and incubated at 4°C overnight. After centrifugation, the precipitates were washed with the lysis buffer and resuspended in 20 μl of Laemmli's sample buffer (62.5 mM Tris–HCl [pH 6.8]), 2% SDS, 10% glycerol, and 0.002% bromophenol blue).

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